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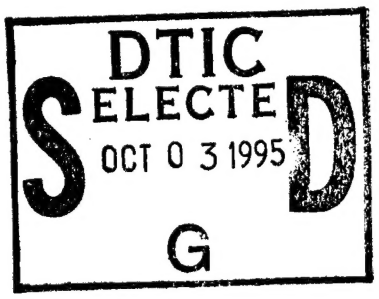

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The independent value of gross and histologic examination, S-phase determination, microvessel density and immunohistochemical expression of epidermal growth factor receptor (egfr), her2/neu, Ki-67 (a proliferation marker), estrogen and progesterone receptor status and p53 accumulation are being determined using a cohort of 1000 women who underwent biopay or mastectomy between 1970 and 1980 and on whom vital status has been obtained. Following accumulation of the complete data set, the relationship between survival and each of these variables will be determined using a proportional hazards model, as will the independent value of each of these prognostic indicators. In addition, the use of a backpropagation neural network model for improving prognostic statements will be explored.

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Timothy Lotz 1 September 1995

PI - Signature Date

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INTRODUCTION

Although the use of adjuvant chemotherapy is now widespread for the treatment of both node-negative and node-positive breast cancer, its use is clearly not justified in all cases. Fully one half of the 182,000 women diagnosed annually with breast cancer will have a prolonged disease free interval following surgical treatment alone. The ten-year recurrence rate for node-negative patients is approximately 30% (1); conversely, about one-third of women with one to three positive lymph nodes treated by surgery alone are recurrence-free at ten years (2). Since these statistics imply that many women can be spared the toxicity of adjuvant chemotherapy, a variety of prognostic indices have been developed to try to identify those women who will be "cured" with surgery alone. Tumor size, grade, nodal status and estrogen/progesterone receptor status are all useful in defining groups of patients with varying risk profiles, but multivariate analyses have yielded inconsistent results as to the independent prognostic value of these markers. Newer markers, such as flow cytometric or immunohistochemical evaluation of proliferative rate, p53 accumulation, epidermal growth factor receptor and erbB2/her2/neu status and microvessel density have all proved promising, but may not have independent value when combined with the more "traditional" markers. "Unfortunately, the studies to date have been too small to evaluate the wide spectrum of prognostic variables simultaneously, leaving both clinicians and patients confused about the tests on which they should rely." (3) Therefore, thorough statistical evaluation of these markers, in conjunction with more traditional clinical and histologic markers, is required to determine if they can provide additional prognostic information within subgroups of patients defined by standard staging and grading methods.

Our investigation will attempt to determine, using neural network methods and multivariate statistical modeling, the independent value of histologic grading, tumor size, lymph node status, flow cytometry, immunohistochemical determination of proliferation, estrogen/progesterone receptor status, p53 accumulation, her2/neu expression, epidermal growth factor receptor (egfr) expression and tumor microvessel density in predicting the long term (greater than ten years) outcome of patients treated before the widespread use of effective adjuvant chemotherapy. This should allow identification of women who may be adequately treated by surgery alone.

BODY

The Armed Forces Institute of Pathology (AFIP) maintains specimens from over 10,000 women with breast cancer. Demographic analysis reveals that most of these specimens originate from women on active duty or from the dependents of active duty or retired service members. The patients represented are similar to those in the general public in terms of age distribution, race and the presence of metastatic disease at the time of initial biopsy. We recently completed a follow-up investigation of one thousand of these patients, to determine vital status. In each case, a minimum of 10 years follow-up

was obtained, making this an ideal group of patients for assessing the utility prognostic markers in assessing disease outcome. Since the women on whom follow-up was obtained were seen prior to the widespread use of adjuvant chemotherapy, we believe that this represents an ideal group by which to study the independent value of breast cancer prognostic markers for predicting long-term survival following surgical therapy or surgery/irradiation.

Characterization and Follow-up

Between January 1 1970 and July 1 1993, slides and paraffin blocks from over 18,000 malignant neoplasms of the breast have been submitted to the AFIP. Slightly under half of these cases represent either unusual histologic variants (such as metaplastic carcinoma), or unusual presentations of breast cancer which led to submission of the case to AFIP for a second opinion. Over half of these cases represent "ordinary" infiltrating carcinomas of the breast which were submitted to the AFIP either as part of quality assurance programs, or in response to a Department of Defense regulation which directed that all malignant neoplasms diagnosed in military hospitals be submitted to the AFIP for histologic confirmation. We have identified within the AFIP database 10647 such cases carrying diagnoses of infiltrating or invasive carcinoma of the breast; 88 percent of these cases represent the dependents of active duty or retired military personnel who have been treated in United States military hospitals. In 3424 of these cases, lymph node metastases were present at the time of case submission. Two hundred and twenty-seven patients are male.

The age and racial/ethnic distributions of the cases are shown in the tables below:

Age	Number
<20	3
20-29	227
30-39	1317
40-49	2717
50-59	2743
60-69	2160
70-79	970
80-89	275
90-99	26
>100	1
Unknown	208

Race	Number
White	6791
Black	660
Asian	149
Pac Islands	40
Native Amer	22
Mixed	4
Unknown	2981

The age, gender and racial/ethnic distributions are similar to those reported elsewhere for breast cancer in the United States, as is the proportion presenting from lymph node metastases. It is apparent, therefore, that the material represented in this collection represents a representative subset of invasive and infiltrating breast carcinomas diagnosed in the United States since 1970.

Follow-up investigation for determination of vital status was performed on 1000 women for whom social security numbers could be obtained from the DEERS database. Vital status was obtained on 99.9% of these women using the National Death Index or the Equifax National Death Search (ENDS).

Overview of Tumor Characterization

The study requires assessment of tumors from each of these 1000 patients for traditional clinical and histological prognostic factors (lymph node status, tumor size, and Elston grade). Following histologic assessment, the S-phase fraction for each of these tumors is determined using flow cytometry. Microvessel density is determined using sections immunohistochemically stained using anti CD-34, to pick up neovascularization. Finally, egfr expression, her2/neu expression, Ki-67 expression, estrogen/progesterone receptor status and p53 accumulation are assessed using immunohistochemical techniques. The prognostic significance of each of these factors will be assessed using univariate statistical methods. The independent prognostic significance of each variable significant in a univariate sense will be determined using stepwise Cox regression and a stepwise logistic regression. Finally, a model for predicting individual patient survival will be developed using a backpropagation neural network algorithm.

A. Histologic Assessment

The examining pathologist reviews the surgical pathology report, and record on a worksheet the reviewing pathologist's measurement of tumor size and number of lymph node metastases. If the tumor is small enough that a cross section can fit entirely on a microscope slide (less than approximately 2.0 cm), the pathologist will remeasure the tumor size from the histologic slides (including the original slides), recording this value separately from that determined by the submitting pathologist.

Following this initial assessment, a thorough microscopic assessment of the tumor is performed. Variables assessed by the pathologist include

- **Necrosis (present or absent)**
- **Lymphatic/vascular permeation (present or absent)**
- **Mitotic figure count (per 10 hpf)**
- **Percent intraductal component (0%, < 25%, GE 25%)**
- **Histologic subtype (ductal, lobular, medullary, etc.)**
- **Nuclear pleomorphism score (uniform well differentiated = 1, intermediate=2, highly variable, anaplastic=3)**
- **Tubule formation score (tubules formed in > 75% of tumor = 1, tubules formed in 10-75% of tumor=2, tubules formed in <10% of tumor=3)**

The mitotic activity index (MAI) is defined for this

study as the total number of mitotic figures in ten adjacent fields in the most cellular region at the tumor periphery, where active growth is most likely (40 objective, numerical aperture 0.65, diameter of one field at specimen level 450 micrometers). To avoid field at specimen level 450 micrometers). To avoid confusing pyknotic nuclei with mitotic figures, only those mitotic figures in which there are clearly defined spicules in cells having no hint of eosinophilia are counted. Care is thus taken to exclude from counts hyperchromatic and apoptotic nuclei, or nuclei from infiltrating lymphocytes (4) .

The tubule formation scores and the percentage of intraductal component are based on the pathologist's estimate of the percentages, considering the complete tumor mass as 100%.

The mitotic figure count, tubule formation score and nuclear pleomorphism score are used to assign a tumor grade using Elston's modification of the Bloom and Richardson grading scheme (5). This grade is assigned by adding the scores for tubule formation, nuclear pleomorphism and mitotic rate (<12/10 hpf=1, 12-22/10 hpf=2, >22/10 hpf=3) to give an aggregate score ranging from 3 to 9. Scores of 3-5 are considered well differentiated, 6 and 7 intermediate, and 8 and 9 poorly differentiated). Previous investigations have demonstrated prognostic value in this grading system, but have not carefully compared it to other grading schemes.

The Elston grade is a component of the Nottingham prognostic index (NPI) which is calculated as

NPI = 0.2 * tumors size (cm)+lymph node state (1-3) + histologic grade (1-3),

where the grade is computed as above and the lymph node stage is defined as 1 for no metastases, 2 for 1-3 positive nodes, and 3 for > 3 positive nodes. This prognostic index will also be computed for each patient.

A third prognostic index, the "morphometric prognostic index" (MPI) (6) is assigned using the mitotic index, the tumor size, and the node status.

MPI = 0.3341 x (MAI)↑1/2↑ + 0.2342 x (tumor size in cm) - 0.7654 x node status

where the lymph node status is given the value 2 if no nodes contained tumor, and 1 if any lymph nodes contain tumor. This index has previously been shown as useful in predicting prognosis as multivariate models including flow cytometric data, estrogen receptor status, menopausal status, and various morphometric features.

The evaluating pathologist will also select three blocks to be further considered for flow cytometry.

B. Selection of Blocks for Flow Cytometry and Molecular Marker Studies

Following initial assessment, the pathologist and a flow cytometrist select a block for flow cytometric and immunohistochemical evaluation. Two 80 micrometer sections are cut for flow cytometry, and 10 six micron sections are cut for immunohistochemical evaluation (half for this study, and half for the associated studies). Many cases accessioned prior to approximately 1981 require re-embedding prior to sectioning, however, as the blocks are not shaped properly to accommodate current microtomes, and the paraffin is frequently too brittle to enable cutting of high quality sections.

C. Histologic Procedures

Sections are cut to 6 micron thickness using a rotary microtome, and floated onto slides which have been slightly frosted so as to permit tissue to remain attached during immunohistochemical staining. Eighty micron sections for flow cytometry.

Specimens are prepared for flow cytometric analysis by the Heiden, Wang and Tribukait (7) adaptation of the Tribukait method. Briefly, the eighty micron sections described above are deparaffinized with xylene and rehydrated through graded (95:5, 80:20, 50:50, 0:100) ethanol/water solutions. Sections are then digested in 1 ml of a 0.1% protease XXIV (Sigma) in 0.1M Tris, 0.07M NaCl, (pH 7.2) solution at 37°C for 2 hours. Following digestion, the sample is placed on a shaker for 30 minutes to dislodge nuclei. Nuclei are then counted on a hemocytometer. If the count is low, 100 microliters of fetal calf serum is added to stop digestion, and the sample is allowed to stand in refrigerator at 4°C overnight. The next morning a repeat cell count is performed, which virtually always reveals an adequate number of nuclei. Following disaggregation, nuclei are stained by adding 1 ml of DAPI-phosphate solution (10 uM DAPI, 800 mM disodium-hydrogenphosphate) or DAPI-citrate solution (10 uM DAPI, 800 mM trisodium citrate) to the cell suspension, at room temperature (22°) for 1 to 8 hours or in refrigerator (4°C) overnight. Following filtration through a nylon mesh the sample is ready for analysis.

D. Flow Cytometry

Following system calibration using fluorescent calibration beads, specimen analysis are carried out on a Coulter Epics Elite flow cytometer using 325 nm light from a helium-cadmium laser to excite the DAPI nuclear stain. Ultraviolet light from the laser is filtered from the emission analysis path by a 844 nm dichroic long pass filter followed by a 525 nm dichroic bandpass filter. Eighty to one hundred nuclei/second are analyzed until signals from 20,000 nuclei have been recorded.

Analysis of flow histograms is carried out using Multicycle (Phoenix Flow Systems, San Diego, CA) cell cycle analysis software. Program options for removing the effects of sectioning debris from the flow histogram (8), and the effects of nuclear "doublets"

and higher order aggregates (9) are utilized for all analyses. Options allowing for one, two or three simultaneous cell cycle series to be considered within the analysis are run; that option giving the best overall fit to the data are the basis for all calculations of S-phase. Aneuploidy is only recorded when a distinct "shoulder" or separate peak is identified on the original histogram; aneuploidy ias not recorded on the sole basis of being able to fit two separate peaks under a single histogram feature using the Multicycle software.

E. Molecular Marker Assays

Immunohistochemical methods are used to assess tumor proliferation, estrogen/progesterone receptor status, p53 accumulation, her2/neu expression, epidermal growth factor receptor (egfr) expression and microvessel density.

Tumor proliferation is assessed by staining with monoclonal antibody MIB-1 (AMAC), which stains the same epitope stained by the Ki-67 antibody, but works in paraffin-embedded sections. Estrogen receptor status is assessed using an anti-estrogen receptor monoclonal antibody (Novacastra); progesterone receptor status is assessed using an anti-progesterone receptor antibody (AMAC). Epidermal growth factor receptor is assessed using a polyclonal antibody available from Oncogene Science. Accumulation of p53 is assessed using monoclonal antibody DO-7 (Novacastra). Her2/neu expression will be assessed using a monoclonal antibody available from Signet. Microvessel density is assessed on sections stained with anti-CD34 (Dako).

Assays for estrogen receptor, p53, egfr, and Ki-67 require use of "antigen retrieval" techniques. Briefly, sections are dewaxed and rehydrated in graded alcohol solutions, then placed in a Coplan jar containing a pH6 citrate buffer. Sections are then heated in a 720 watt microwave oven for 5 minutes; distilled water is added to keep slides covered and the slides are then heated for 5 more minutes.

Following 20 minutes of cooling, the slides are washed with tris buffered saline prior to staining.

Immunocytochemical assays are performed using the ABC technique. Following deparaffinization and rehydration, slides are incubated in a normal serum blocking agent overnight at 4↑o↑C. Endogenous peroxidase activity is blocked using 10% hydrogen peroxide in methanol, then the slides are washed. Incubation by primary antibody and washing follows, followed by incubation with secondary antibody and another wash step. Finally, ABC complex is added and a diaminobenzidine substrate is developed. Following a final washing and dehydration, the slides are coverslipped.

Angiogenesis is quantitated using the technique proposed by Weidner and coworkers (10). Quantitative assessment of the other immunohistochemical assays is attained by a cell-counting technique (11).

F. Data Archiving

Data obtained in this study is maintained in a relational database which has been developed using Microsoft Access. This database now contains the records of all women whose biopsies were seen at the AFIP from 1970-1980, and on whom paraffin tumor blocks are available. Histologic analysis, flow cytometry results and immunohistochemical results are linked to this database using the AFIP accession number.

G. Statistical Analysis

The univariate prognostic significance of each group will be assessed by computing Kaplan-Meier survival curves together with the associated logrank and generalized Wilcoxan statistics. Those which are univariate significant will then be included in a multivariate model and in a neural network analysis. Independent value of univariate significant prognostic factors will be determined using the Cox life table regression model. In addition, the logistic regression model is used to develop predictors of ten-year survival for individual patients, as

will a backpropagation neural network algorithm. Analyses are performed separately for node-negative and node positive women further stratified into two additional subgroups on the basis of tumor size either less than two cm. or equal to or greater than 2 cm. Power analysis suggests that the above analysis will be capable of detecting (with a power of 0.9 or higher) prognostic factors able to discern a two-fold relative risk in each of the four subgroups defined above.

CONCLUSIONS

To date, 700 cases have been histologically examined, and blocks selected for immunocytochemical and flow cytometric assessment. Of these, 440 have been cut, and slides prepared for flow cytometry and immunohistochemical study. Flow cytometric analysis has been completed on 275 of these cases.

Two hundred of these cases have been identified for inclusion in a separate study which will correlate loss of heterozygosity on several chromosomes with clinical and prognostic factor assessment.

In 54 of these cases, there is insufficient material from the primary tumor for immunohistochemical and flow cytometric assessment.

Data analysis will not be carried out until all flow cytometric and immunohistochemical investigations have been completed. For this reason, no conclusions are available at this time.

Completion of this study has been delayed due contracting difficulties; nevertheless, we believe that the study can be substantially completed within the calender year.

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